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PROTEIN KINASES

This invention is related to the one or more inventions described in British Patent Applications Nos. 9224057.1, filed 17th November 1992; 9304677.9 and 9304680.3, both filed 8th March 1993; 9311047.6, filed 28th May 1993; and 9313763.6, filed 2nd July 1993. In particular, this invention relates to nucleotides, proteins obtained by expression therefrom and antibodies raised to peptides derived from the sequence, e.g. by the means described in the Application No. 9304680.3.

A particularly important aspect of the invention is based on the discovery that a member of the serine/threonine kinase family is a TGF-ß-type I receptor. It has not previously been possible to isolate this receptor, to allow determination of its sequence or of the corresponding gene, as the expression levels of the receptor are very low.

The isolation of the nucleic acid molecule which codes for the TFG-ß-type I receptor allows it to be expressed at high enough levels in cell lines, e.g. for use in antagonist/agonist screening. It also allows the production of the protein, and portions of the protein, e.g. the extracellular domain, for therapeutic, diagnostic and other commercial purposes.

Summary

A cDNA clone encoding a 53 kd serine/threonine kinase receptor with an overall structure similar to that of the type II receptor for TGF-β was obtained. ^{125}I-TGF-β1 bound to porcine endothelial cells transfected with the cDNA, and formed a cross-linked complex of 70 kd, characteristic of a TGF-β type I receptor. Immunoprecipitation of the cross-linked complexes by antibodies against the cloned receptor revealed the 70 kd complex as well as a 94 kd TGF-β type II receptor complex. The immunoprecipitated novel serine/threonine kinase receptor had biochemical properties of the TGF-β type I receptor, and was observed in TGF-β responsive cells. Transfection of the cloned cDNA into TGF-β type I receptor deficient cells restored TGF-β-induced PAI-1 production. These results suggest that signal transduction by TGF-β involves the formation of a heteromeric complex of two different serine/threonine kinase receptors.

Introduction

Transforming growth factor-β (TGF-β) is a family of multifunctional proteins that act on many different types of cells (for reviews see Roberts and Sporn, 1990; Moses et al., 1990). TGF-β was first identified as a 25 kd homodimer, that stimulated the growth of normal rat fibroblasts in soft agar culture (Assoian et al., 1983). However, it was later shown to be a potent growth inhibitor for most cell types (Moses et al., 1985). TGF-β also has many other biological effects, including regulation of cell differentiation, stimulation of extracellular matrix formation, and modulation of the immune response. Three different isoforms of TGF-β, denoted TGF-β1, -β2, and -β3, with similar but not identical biological activities (Ohta et al., 1987; Cheifetz et al., 1990), have been identified in various mammalian tissues and cells. TGF-βs belong to a larger family of structurally related proteins, which includes activins and inhibins (Vale et al., 1990), bone morphogenetic proteins (BMPs) (Wozney et al., 1988), and Müllerian inhibiting substance (Cate et al., 1986). The proteins of the TGF-β superfamily have a wide variety of biological activities and play important roles in the morphogenesis, e.g. during different stages of development (Akhurst et al., 1991; Lyons et al., 1991).

TGF-βs exert their effects through binding to specific cell surface receptors. By affinity labeling and cross-linking by radioiodinated TGF-βs, a number of TGF-β receptors (or binding proteins) have been identified, including type I (53 kd), type II (75 kd) and type III receptors (or betaglycan, 300 kd), which are found in most cells (reviewed in Massagué, 1992; Lin and Lodish, 1993; Miyazono et al., 1993). The type I and the type II receptors are the most important for signal transduction (Segarini et al., 1989; Boyd and Massagué, 1989; Laiho et al., 1990).

The TGF-β type III receptor is a membrane proteoglycan lacking cytoplasmic protein kinase domain (López-Casillas et al., 1991; Wang et al.,

1991; Morén et al., 1992). The transmembrane and intracellular parts of the type III receptor are very similar to the corresponding region of endoglin, a 18C kd dimeric protein, which is expressed in endothelial cells and in the mesangium of the kidney (Gougos and Letarte, 1988; 1990). Endoglin binds TGF-β1 and -β3, but not TGF-β2, with high affinities, whereas the type III receptor binds all three mammalian isoforms of TGF-β (Cheifetz et al., 1992). The type III receptor and endoglin may be indirectly involved in the signal transduction, e.g. by presenting the ligands to the type II and type I receptors (Wang et al., 1991; Cheifetz et al., 1992).

The TGF-β type II receptor cDNA was obtained by an expression cloning strategy (Lin et al., 1992). Similar to the activin type II and type IIB receptors (Mathews and Vale, 1991; Attisano et al., 1992; Mathews et al., 1992), the TGF-β type II receptor has a serine/threonine kinase domain in its cytoplasmic portion. Autophosphorylation on serine and threonine residues of the TGF-β type II receptor kinase has been demonstrated using a bacterial fusion protein (Lin et al., 1992). A transmembrane serine/threonine kinase had previously been found in the Daf-1 protein (Georgi et al., 1990), which is involved in the dauer larva development. These results suggest that receptor serine/threonine kinases form a new receptor family, which may include the receptors for the proteins in the TGF-β superfamily.

The TGF-β type I receptor has been shown to be a 53 kd glycoprotein using affinity cross-linking techniques (Cheifetz et al., 1988a; Cheifetz and Massagué, 1991). Studies using chemically-induced TGF-β receptor mutant cells indicated that both type I and type II receptors are indispensable for TGF-β signaling (Boyd and Massagué, 1989; Laiho et al., 1990); the type II receptor is needed for the binding of TGF-β to the type I receptor, and the type I receptor is required for the signal transduction induced by the type II receptor (Wrana et al., 1992). Very recently, a serine/threonine kinase receptor termed Tsk 7L has been reported, which has a size similar to a TGF-β type I receptor

and binds TGF-β in the presence of the TGF-β type II receptor (Ebner et al., 1993). However, the structural and functional properties of the Tsk 7L has not been fully determined.

Here, we report the isolation and characterization of a human cDNA clone encoding a novel 53 kd putative serine/threonine kinase receptor. The biochemical and biological properties of the cloned receptor indicate that it represents a type I receptor for TGF-β.

Results

Cloning and Analysis of an ALK-5 cDNA

In order to identify novel serine/threonine kinase receptors, a PCR-based approach was used with the prediction that these receptors share sequence similarities to each other (ten Dijke et al., 1993). PCR was performed using cDNA from human erythroleukemia (HEL) cells and degenerated primers that were based on conserved regions in the serine/threonine kinase receptors. The primers used were derived from subdomain II (nomenclature according to Hanks et al., 1988), which includes the lysine residue involved in ATP binding, and from subdomain VIII, which is suggested to determine the hydroxyamino acid specificity for serine and threonine residues rather than tyrosine residues (Hanks et al. 1988). Several different PCR products were obtained, including the TGF-β type II receptor and the activin type II receptor. One PCR recombinant, 11.1, encoded a novel amino acid sequence (not shown), and was therefore used to screen a random primed HEL cell λgt10 cDNA library. Screening of about 1 x 10^5 independent clones yielded one positive clone, which has an insert size of 5.3 kb with two internal EcoRI sites (Figure 1A). Since this clone was obtained as the fifth clone in our cloning of novel serine/threonine kinase receptors, it was denoted activin receptor-like kinase

(ALK)-5. Nucleotide sequencing revealed an open reading frame of 1509 bp, coding for 503 amino acids. The open reading frame is flanked by a 5' untranslated sequence of 76 bp, and a 3' untranslated sequence of 3.7 kb which was not completely sequenced. The nucleotide and deduced amino acid sequences of ALK-5 are shown in Figure 1B. The amino acid sequence has a 90% sequence identity to the deduced amino acid sequence of the 11.1 PCR recombinant that was used as a probe for screening of the library.

Structure of the ALK-5 Protein

In the 5' part of the open reading frame, only one ATG codon was found; this codon fulfills the rules for initiation of translation (Kozak et al., 1987). An in-frame stop codon was found at nucleotides (-54)-(-52) in the 5' untranslated region. The predicted starting ATG codon is followed by a stretch of hydrophobic amino acid residues which has the characteristics of a cleavable signal sequence. Therefore, the first ATG codon is likely to be used as a translation initiation site. A preferred cleavage site for the signal peptidase, according to von Heijne (1986), is located between amino acid residues 24 and 25. The calculated molecular weight of the primary translated product of the ALK-5 without signal sequence is 53,646.

Another hydrophobic region, which represents a putative transmembrane domain (Kyte and Doolittle, 1982), is found at amino acid residues 126-147, followed by basic residues at His-149 and Arg-151, as is common for the cytoplasmic side of transmembrane regions (Weinstein et al., 1982). The extracellular domain has a cysteine-rich region with one potential N-glycosylation site. It has little sequence similarity with the TGF-β type II receptor (Figure 1C), activin type II and IIB receptors, and Daf-1 (less than 17 % amino acid sequence identity). However, a fair alignment of nine of the ten cysteine residues could be performed. The cytoplasmic part has a putative protein kinase domain, which contains the consensus motifs specific for

serine/threonine kinases, and is 41% identical to that of the TGF-β type II receptor (Figure 1B and C, see Discussion).

Similar to the type II receptors for activin and TGF-β, the protein kinase domain of ALK-5 is interrupted by short streches of amino acid residues between subdomains VIA and VIB and between subdomains X and XI (Figure 1B and C). Based on the sequence similarity with the type II receptors for TGF-β and activin, the C-terminus of the kinase domain of ALK-5 was set at Gln-498, and thus the C-terminal tail of ALK-5 has only 5 amino acid residues, which is considerably shorter than those of the TGF-β and activin type II receptors (more than 23 amino acid residues).

The ALK-5 cDNA Encodes a 53 kd Glycoprotein

The ALK-5 cDNA was subcloned into the SV40-based expression vector pSV7d and transfected into COS-1 cells. Two days after transfection, the cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine and subjected to immunoprecipitation using an antiserum termed VPN, which was raised against a synthetic peptide corresponding to amino acids 158-179 in the juxtamembrane part of the cytoplasmic domain. This region is divergent in sequence between the various serine/threonine kinase receptors (not shown). A component of 53 kd was seen when the samples were analyzed by SDS-polyacrylamide gel electrophoresis (Figure 2). This component was not seen when preimmune serum was used, or when 10 μg of blocking peptide was added together with the antiserum. Moreover, it was not detectable in samples derived from untransfected COS-1 cells using either preimmune serum or the antiserum. When the immunoprecipitated sample was treated with endoglycosidase F, that hydrolyzes the complex- and high mannose-types of N-linked carbohydrates, the 53 kd band shifted to 51 kd. The extracellular domain of ALK-5 contains one potential acceptor site for N-glycosylation and

the size of the deglycosylated protein is close to the predicted size of the core protein.

Binding of ^{125}I-TGF-β1 to ALK-5 in Transfected PAE Cells

In order to investigate whether the ALK-5 cDNA encodes a receptor for TGF-β, porcine aortic endothelial (PAE) cells were transfected with a cytomegalovirus (CMV)-based expression vector pcDNA I/NEO containing the ALK-5 cDNA, and analyzed for the binding of ^{125}I-TGF-β1, followed by affinity cross-linking and SDS-gel electrophoresis. ^{125}I-TGF-β1 formed a 70 kd cross-linked complex in the transfected PAE cells (PAE/TβR-I cells). The size of this complex was very similar to that of the TGF-β type I receptor complex observed at lower amounts in the untransfected cells (Figure 3A). A concomitant increase of a 94 kd TGF-β type II receptor complex, could also be observed in the PAE/TβR-I cells. Components of 150-190 kd, which could represent cross-linked complexes between the type I and type II receptors (see Discussion), were also observed in the PAE/TβR-I cells.

In order to determine whether the cross-linked 70 kd complex contained the protein encoded by the ALK-5 cDNA, the affinity cross-linking was followed by immunoprecipitation using the VPN antiserum. A 70 kd cross-linked complex could be precipitated by the VPN antiserum in PAE/TβR-I cells, and a weaker band of the same size was also seen in the untransfected cells (Figure 3A), indicating that the untransfected PAE cells contained a low amount of ALK-5. The 70 kd complex was not observed when preimmune serum was used (Figure 3A), or when immune serum was blocked by 10 μg of peptide (not shown). Moreover, a coprecipitated 94 kd component could also be observed in the PAE/TβR-I cells. The latter component is likely to represent a TGF-β type II receptor complex, since an antiserum termed DRL, which was raised against a synthetic peptide from the C-terminal part of the TGF-β type II receptor, precipitated a 94 kd TGF-β type II receptor complex, as well as a 70

kd type I receptor complex from PAE/TβR-I cells (Figure 3B). Based on these results, as well as the observations which will be presented below, we conclude that ALK-5 encodes a type I receptor for TGF-β.

The carbohydrate contents of ALK-5 and the TGF-β type II receptor were characterized by deglycosylation using endoglycosidase F on cross-linked and immunoprecipitated ^{125}I-TGF-β1 receptor complexes from the PAE/TβR-I cells. The ALK-5 cross-linked complex shifted from 70 kd to 66 kd, whereas that of the type II receptor shifted from 94 kd to 82 kd (Figure 4A). The observed larger shift of the type II receptor band compared with that of the ALK-5 band is consistent with the deglycosylation data of the type I and type II receptors on rat liver cells reported previously (Cheifetz et al., 1988a), and fits well with the facts that the porcine TGF-β type II receptor has two N-glycosylation sites (Lin et al., 1992), whereas ALK-5 has only one (Figure 1B).

Binding of TGF-β1 to the type I receptor is known to be abolished by transient treatment of the cells with dithiothreitol (DTT) (Cheifetz and Massagué, 1991; Wrana et al., 1992). When analyzed by affinity cross-linking, binding of ^{125}I-TGF-β1 to ALK-5, but not to the type II receptor, was completely abolished by DTT treatment of PAE/TβR-I cells (Figure 4B). Affinity cross-linking followed by immunoprecipitation by the VPN antiserum showed that neither the ALK-5 nor the type II receptor complexes were precipitated after DTT treatment, indicating that the VPN antiserum reacts only with ALK-5 (Figure 4B). In contrast, the DRL antiserum precipitated only the type II receptor complex after DTT treatment (data not shown). In conclusion, our data show that the VPN antiserum recognizes a TGF-β type I receptor, and that the type I and type II receptors form a heteromeric complex.

Expression of the ALK-5 mRNA in Different Tissues

The ALK-5 mRNA size and distribution were determined by Northern blot analysis. Figure 5 shows that a 5.5 kb mRNA is expressed in all human

tissues tested, being most abundant in placenta and least in brain and heart. The size of the transcript suggests that the obtained cDNA clone was close to full length.

Expression of the ALK-5 Protein in Different Cell Types

Several cell lines were tested for the expression of the ALK-5 protein by cross-linking followed by immunoprecipitation using the specific antisera against ALK-5 and the type II receptor. The mink lung epithelial cell line, Mv1Lu, is widely used as target cells for TGF-β action and well characterized regarding TGF-β receptors (Laiho et al., 1990; 1991a). The VPN antiserum precipitated both type I and type II TGF-β receptors in the wild type Mv1Lu cells (Figure 6A). The DRL antiserum also precipitated components with the same size as those precipitated by the VPN antiserum. A mutant cell line, which lacks the TGF-β type I receptor and does not respond to TGF-β (R mutant, Laiho et al., 1990, 1991a), was also investigated by cross-linking followed by immunoprecipitation. Consistent with the results obtained by Laiho et al. (1990), the type III and type II TGF-β receptor complexes, but not the type I receptor complex, were observed by affinity cross-linking (Figure 6A). Cross-linking followed by immunoprecipitation using the DRL antiserum revealed only the type II receptor complex, whereas neither the type I nor type II receptor complexes were seen using the VPN antiserum. When the cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine and subjected to immunoprecipitation using the VPN antiserum, the 53 kd ALK-5 protein was precipitated in both the wild type and R mutant Mv1Lu cells (data not shown). These results suggest that the type I receptor expressed in the R mutant is ALK-5, which has lost the affinity for binding to TGF-β after mutation.

The type I and type II TGF-β receptor complexes could be precipitated by the VPN and DRL antisera in other cell lines, including human foreskin fibroblasts (AG1518), human lung adenocarcinoma cells (A549), and human

oral squamous cell carcinoma cells (HSC-2) (Figure 6B). Affinity cross-linking studies revealed multiple TGF-β type I receptor-like complexes of 70-77 kd in these cells. These components were less efficiently competed by excess unlabeled TGF-β1 in HSC-2 cells. Moreover, the type II receptor complex was low or not detectable in A549 and HSC-2 cells. Cross-linking followed by immunoprecipitation revealed that the VPN antiserum precipitated only the 70 kd complex among the 70-77 kd components. The DRL antiserum precipitated the 94 kd type II receptor complex as well as the 70 kd type I receptor complex in these cells, but not the putative type I receptor complexes of slightly larger sizes. These results suggest that multiple type I TGF-β receptors may exist and that the 70 kd complex containing ALK-5 forms a heteromeric complex with the TGF-β type II receptor cloned by Lin et al. (1992) more efficiently than the other species. In rat pheochromocytoma cells (PC 12) which have been reported to have no TGF-β receptor complexes by affinity cross-linking (Massagué et al., 1989), neither VPN nor DRL antisera precipitated the TGF-β receptor complexes (data not shown).

Restoration of TGF-β Responsiveness by Transfection of the ALK-5 cDNA

The question of whether ALK-5 is a functional TGF-β type I receptor was investigated by transfection of the cDNA into the R mutant clone of Mv1Lu cells. Wild type Mv1Lu cells responded to TGF-β and produced plasminogen activator inhibitor-1 (PAI-1), whereas the R mutant clone did not produce PAI-1 even after the stimulation by TGF-β1 (Figure 7), as previously described (Laiho et al., 1990; 1991a). Transient transfection of the ALK-5 cDNA into the R mutant clone led to the production of PAI-1 in response to the stimulation by TGF-β1 (Figure 7), indicating that the ALK-5 cDNA encodes a functional TGF-β type I receptor.

Discussion

In the present report, we show the structure of a novel putative serine/threonine kinase receptor, ALK-5. The following observations support the notion that ALK-5 is a type I receptor for TGF-β. i) The size of ALK-5 immunoprecipitated from metabolically labeled COS-1 cells (Figure 2) or PAE/TβR-I cells (data not shown) was 53 kd, which is consistent with the predicted size of a type I receptor (Cheifetz et al., 1988a). ii) ^{125}I-TGF-β1 formed a cross-linked complex of 70 kd, which could be precipitated by an antiserum against ALK-5 from PAE/TβR-I cells (Figure 3). This is the expected size for a TGF-β type I receptor complex (Cheifetz et al., 1988a). iii) A complex of 94 kd, i.e. the expected size of a TGF-β type II receptor complex was coimmunoprecipitated with the 70 kd complex (Figure 3). Moreover, an antiserum raised against the TGF-β type II receptor precipitated complexes of 94 kd as well as the 70 kd, i.e. the same sizes as those precipitated by the antiserum against ALK-5. These results are consistent with the notion that type I and type II receptors form a heteromeric complex after ligand binding (Wrana et al., 1992; Inagaki et al., 1993). iv) Treatment with endoglycosidase F led to a small decrease in size of the 70 kd complex, but to a larger decrease of the 94 kd type II receptor complex (Figure 4A), consistent with previous observations on type I and type II receptors (Cheifetz et al., 1988a), and with the facts that ALK-5 has one potential N-glycosylation site, whereas the porcine type II receptor has two (Lin et al., 1992). v) Treatment of PAE/TβR-I cells with DTT led to loss of binding of ^{125}I-TGF-β1 to ALK-5, whereas the binding to the type II receptor was retained (Figure 4B). This is consistent with the previously described properties of the type I and type II TGF-β receptor complexes (Cheifetz and Massagué, 1991). vi) The distribution of mRNA for ALK-5 was found to be ubiquitous, as is expected for that of the TGF-β type I

receptor. vii) Cross-linked TGF-β type I complexes could be immunoprecipitated by the ALK-5 antiserum from other cell types, including Mv1Lu cells. Moreover, a TGF-β type II receptor antiserum precipitated components of the same sizes as those precipitated by the VPN antiserum from these cells. viii) TGF-β type I receptor deficient cells (R mutant) responded to TGF-β1 by PAI-1 production after the transfection of the ALK-5 cDNA.

Two other TGF-β receptors with sizes in the same range as ALK-5 have been described. One is the TGF-β type IV receptor (70-74 kd cross-linked complex). However, this receptor is expressed only in the pituitary tumor cells (Cheifetz et al., 1988b), and it is thus unlikely that it is the same as ALK-5. The other is a 40 kd component purified from porcine uterus (Ichijo et al., 1991). However, this molecule was recently cloned and was shown to have a different sequence (Ichijo et al., 1993). Based on these observations, we conclude that the ALK-5 cDNA encodes a type I receptor for TGF-β.

The size, susceptibility to endoglycosidase F treatment, and the lack of susceptibility to DTT, support the notion that the coimmunoprecipitated 94 kd receptor by the VPN antiserum is the type II receptor. Complexes of 150-190 kd were also observed in the PAE/TβR-I cells, which were coimmunoprecipitated by the VPN antiserum (Figure 3). It is possible that these components represent cross-linked complexes between the type I and type II receptors. Alternatively, the components may represent other types of TGF-β receptors, e.g. endoglin, which is expressed in endothelial cells (Cheifetz et al., 1992).

Apart from the transfected PAE cells, the TGF-β type I receptor could also be immunoprecipitated from parental PAE cells (Figure 3A), wild type Mv1Lu cells, human foreskin fibroblasts, A549 cells, and HSC-2 cells (Figure 6). The TGF-β type I and type II receptor complexes observed in Mv1Lu cells using ^{125}I-TGF-β2 and -β3 could also be precipitated by the VPN and DRL

antisera (data not shown). Similar to the TGF-β type II receptor (Lin et al., 1992, Wrana et al., 1993), TGF-β2 bound to the type I receptor less efficiently than TGF-β1 and -β3. These results, together with the data obtained by Northern blot analysis, suggest that ALK-5 is widely expressed and serves as a TGF-β type I receptor in many cell types. However, this does not rule out the possibility that there exist other molecules that can function as type I receptors for TGF-β.

By affinity cross-linking studies, some carcinoma cells were found to express predominantly TGF-β type I receptor-like components, with a low (or no) level of the type II receptor complex (Figure 6B, and Ohta et al., 1987; Geiser et al., 1992; Ebner et al., 1993). The present report revealed that certain components of 70-77 kd do not form a heteromer with the TGF-β type II receptor (Figure 6B), and may therefore have different functional properties compared to those of ALK-5. The intensities of the type I and type II receptor bands observed in cross-linking followed by immunoprecipitation, were different in various cell types (Figures 3 and 6). In A549 cells, antisera against type I as well as type II receptors predominantly precipitated the type I receptor complex with only low levels of the type II receptor complex; it is thus possible that the efficiency of cross-linking of the type I receptor is higher than that of the type II receptor.

Using a PCR-based approach, we recently obtained four additional serine/threonine kinase receptors, termed ALK-1 to -4 (ten Dijke et al., 1993). The five ALKs have similar sizes and 60-90% amino acid sequence identities to each other in their kinase domains, whereas they have 37-42% identities to the activin type II and IIB receptors and to the TGF-β type II receptor. Thus, the ALKs form a subfamily among the serine/threonine kinase receptors. Their sizes and sequence similarities make them interesting candidates for being additional type I receptors for TGF-β1 or for other TGF-β isoforms, activins, inhibins, BMPs or other members of the TGF-β superfamily. After the original

submission of this manuscript, Ebner et al. (1993) reported that a mouse serine/threonine kinase receptor (Tsk 7L) bound TGF-β and formed a TGF-β type I receptor-like complex with a slightly higher molecular weight compared to the endogenous human type I receptor complex. The Tsk 7L clone is the mouse counterpart of the human ALK-2 (ten Dijke et al., 1993), which was also reported as SKR1 by Matsuzaki et al. (1993). The Tsk 7L clone needs the type II TGF-β receptor for ligand-binding; however, the physical interaction of the Tsk 7L clone with the type II receptor has not been fully elucidated (Ebner et al., 1993). Whether the Tsk7L clone and ALK-5 have similar functions or not, will be the aim of the future studies.

Cytokine receptors form multimeric complexes upon ligand binding, and some of the receptors, e.g. gp130, are shared between different cytokines (reviewed in Nicola and Metcalf, 1991; Miyajima et al., 1992). Analogously, some of the serine/threonine kinase receptors may be shared by members of the TGF-β superfamily. However, ALK-5 did not act as a type I receptor for activin; when a cDNA containing the extracellular domain of activin type II receptor was transfected into PAE cells, the endogenous activin type I receptor bound ^{125}I-activin, but the activin type I receptor complex in these cells could not be precipitated by the VPN antiserum (Yamashita, H., unpublished observation).

Studies using chemically-mutagenized Mv1Lu cells, which are resistant to the TGF-β action, have indicated that the type I and type II receptors form a heteromeric complex and are directly involved in the signal transduction of TGF-β (Boyd and Massagué, 1989; Laiho et al., 1990). Two types of TGF-β receptor mutant cells were obtained; the class R mutants showed normal binding to the type II receptor but lacked the binding to the type I receptor, whereas the class DR mutants showed no or anomalous binding to both type I and type II receptors. Somatic cell hybrids between the class R and DR mutants resulted in the restoration of the type I receptor binding (Laiho et al.,

1991a), indicating that the type I receptor is present in the class DR mutants, but the type II receptor is needed for the ligand binding to type I receptor. More recently, the formation of a heteromeric complex between the type I and type II receptors was demonstrated by the observations that the type I receptor was coimmunoprecipitated with the type II receptor, and that transfection of the type II receptor into class DR mutants rescued the TGF-β binding to the type I receptor and made the cells responsive to TGF-β (Wrana et al., 1992). The properties of ALK-5 are fully consistent with the previous conclusions regarding the TGF-β type I receptor and the activated TGF-β receptor complex. Thus, formation of the heteromeric complex of two serine/threonine kinase receptors may be an important event for TGF-β signaling in its target cells. Future studies will be aimed at exploring the exact mechanism for activation of the TGF-β receptor complex and to determine the contribution of each one of the two receptors in the activation of downstream components in the signal transduction pathway. Furthermore, it will be important to determine whether the receptor complex is a heterodimer or a heterotetramer of TGF-β type I and type II receptors and whether additional TGF-β receptors, e.g. the type III receptor and/or endoglin, are also part of the complex.

Experimental Procedures

Preparation of mRNA and Construction of a cDNA Library

For construction of a cDNA library, poly(A)+RNA was isolated from a human erythroleukemia cell line, HEL (American Type Culture Collection), by the guanidinium isothiocyanate method (Chirgwin et al., 1979), followed by use of a polyAT tract mRNA isolation kit (Promega). The isolated mRNA was used for the synthesis of random primed (Amersham) cDNA. A λgt10 cDNA library with

$1x10^5$ independent cDNA clones was prepared by RiboClone cDNA synthesis system (Promega) and in vitro packaging kit (Amersham).

Generation of a cDNA Probe by PCR

For the generation of cDNA probes by PCR (Lee et al., 1988), degenerated PCR primers were constructed based upon the amino acid sequence similarity between the mouse activin type II receptor (Mathews and Vale, 1991) and Daf-1 (Georgi et al., 1990) in the kinase domains II and VIII. Oligonucleotides were synthesized using Gene assembler plus (Pharmacia-LKB). The sense primer, B3-S, was a 25-mer oligonucleotide (5'-GCG GATCCGT(C/G/T)GC(A/C/T)GT(C/G/T)AA(A/G)AT(A/C/T)TT) derived from the conserved motif (VAVKIF in single letter code) in subdomain II with a 5' BamHI restriction enzyme site. The antisense primer, E8-AS, was a 20-mer oligonucleotide (5'-CGGAATTC(A/G/T)GG(A/G/T)GCCAT(A/G)TA) derived from the conserved sequence (YMAPE) in subdomain VIII with a 5' EcoRI site. PCR was performed in a 100 µl volume using first strand cDNA prepared from HEL mRNA, Taq polymerase (Perkin Elmer Cetus) and the oligonucleotide primers. The following program was used for PCR amplification; first 5 thermal cycles, each composed of 94°C (1 min), 50°C (1 min), 55°C (2 min), 72°C (1 min) followed by 20 thermal cycles of 94°C (1 min), 55°C (0.5 min), 72°C (1 min). A second round of PCR was performed with 3 µl of the first reaction as a template. After 25 thermal cycles, each composed of 94°C (1 min), 55°C (0.5 min), 72°C (1 min), the products with the expected sizes (~460 bp) were purified by agarose gel electrophoresis. The PCR products were ligated into pUC19 (Yanisch-Perron et al., 1985) at BamHI/EcoRI sites, and nucleotide sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using T7 DNA polymerase (Pharmacia-LKB). One of the PCR recombinants denoted 11.1 showed a novel sequence and was used for isolation of a full-length cDNA.

Isolation and Characterization of a cDNA Clone

The HEL cell cDNA library was screened with the insert of PCR recombinant 11.1 labeled by the Megaprime DNA labeling system (Amersham). Hybridization to nitrocellulose filters (Hybond-C extra, Amersham) was performed in 50% formamide, 5 x SSC (1 x SSC is 15 mM sodium citrate and 150 mM sodium chloride), 50 mM sodium phosphate, pH 6.9, 5 x Denhardt's solution, 0.1% SDS and 0.1 mg/ml salmon sperm DNA at 37°C overnight. The filters were washed in 0.5 x SSC, 0.1% SDS at 55°C three times for 15 min, dried and exposed to Fuji X-ray films. Purification of a positive bacteriophage plaque was performed as described by Sambrook et al. (1989). A clone denoted EMBLA was identified and subcloned into pBluescript SK (Stratagene) and thereafter sequenced on both strands. Compressions of nucleotides were resolved using 7-deaza-GTP (United States Biochemical Corp.).

Antibodies

Rabbit antisera against ALK-5 denoted VPN were prepared against a synthetic peptide corresponding to amino acid residues 158-179. Antisera against the TGF-β type II receptor denoted DRL were prepared from a synthetic peptide corresponding to amino acid residues 245-266 (Lin et al., 1992). The peptides were synthesized with an Applied Biosystems 430A Peptide Synthesizer using t-butoxycarbonyl chemistry and purified by reversed phase high performance liquid chromatography. The peptides were coupled to keyhole limpet hemocyanin (Calbiochem-Behring) using glutaraldehyde, as described by Gullick et al. (1985). The coupled peptides were mixed with Freund's adjuvant and used to immunize rabbits.

Transient Transfection of the ALK-5 cDNA

COS-1 cells (American Type Culture Collection) and R mutant of Mv1Lu cells (clone 4-2, a gift from Marikki Laiho and Joan Massagué) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and antibiotics in 5% CO_2 atmosphere at 37°C. The ALK-5 cDNA (nucleotides (-76)-2232), which includes the whole coding region, was cloned into the pSV7d vector (Truett et al., 1985), and used for transfection. Transfection into COS-1 cells was performed by the calcium phosphate precipitation method (Wigler et al., 1979). Briefly, cells were seeded into 6-well cell culture plates at a density of 5×10^5 cells/well, and transfected the following day with 10 µg of plasmid. After overnight incubation, cells were washed three times with a buffer containing 25 mM Tris-HCl, pH 7.4, 138 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.6 mM Na_2HPO_4, and then incubated with Dulbecco's modified Eagle's medium containing 10% FBS and antibiotics. Two days after transfection, the cells were used for metabolic labeling and immunoprecipitation. Transfection into the R mutant clone was performed by a diethylaminoethyl-dextran transfection method (Stratagene) with 0.4 µg DNA as described (Wrana et al., 1992) or by transfection with transfectam reagent (Promega) using 2 µg DNA and 4 µl transfectam reagent. After 48-72 h of transfection, cells were assayed for the PAI-1 production.

Establishment of PAE Cell Lines Expressing ALK-5

PAE cells were cultured in Ham's F-12 medium supplemented with 10 % FBS and antibiotics (Miyazono et al., 1988). The ALK-5 cDNA was cloned into the pcDNA I/NEO vector (Invitrogen), and transfected into PAE cells by electroporation. After 48 h, selection was initiated by adding Geneticin (G418 sulphate; Gibco-BRL) to the culture medium at a final concentration of 0.5 mg/ml (Westermark et al., 1990). Several clones were obtained, and after

analysis by immunoprecipitation using the VPN antiserum, one clone denoted PAE/TβR-I was chosen and further analyzed.

Metabolic Labeling, Immunoprecipitation and SDS-gel Electrophoresis

Metabolic labeling of cells was performed for 6 h in methionine- and cysteine-free MCDB 104 medium with 150 μCi/ml of [^{35}S]methionine and [^{35}S]cysteine (In vivo labeling mix; Amersham). After labeling, the cells were washed with 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, and then solubilized with a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1.5% Trasylol (Bayer) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). After 15 min on ice, the cell lysates were pelleted by centrifugation, and the supernatants were cleared one time with preimmune serum. Samples (1 ml) were then incubated with either 7 μl of preimmune serum or the VPN antiserum for 1.5 h at 4°C. For blocking, 10 μg of peptide was added together with the antiserum. Immune complexes were then given 50 μl of a protein A-Sepharose (Pharmacia-LKB) slurry (50% packed beads in 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.2% Triton X-100) and incubated for 45 min at 4°C. The beads were spun down and washed four times with a washing buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, 1% deoxycholate and 0.2% SDS), followed by one wash in distilled water. The immune complexes were eluted by boiling for 5 min in the SDS-sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS) in the presence of 10 mM DTT, and analyzed by SDS-gel electrophoresis using 7-15% polyacrylamide gels (Blobel and Dobberstein, 1975). Gels were fixed, incubated with Amplify (Amersham) for 20 min, and subjected to fluorography.

Digestion with Endoglycosidase F

Samples from the metabolically labeled COS-1 cells transfected with the ALK-5 cDNA or from PAE/TβR-I cells which were subjected to affinity cross-linking with ^{125}I-TGF-β1 (see below), were immunoprecipitated by the VPN antiserum. The samples were then incubated with 0.5 U of endoglycosidase F (Boehringer Mannheim Biochemica) in a buffer containing 100 mM sodium phosphate, pH 6.1, 50 mM EDTA, 1% Triton X-100, 0.1% SDS and 1% β-mercaptoethanol at 37°C for 24 h. Samples were eluted by boiling for 5 min in the SDS-sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorograpy for the metabolically labeled cells or autoradiography for the cross-linked cells.

Iodination of TGF-β1, Binding and Affinity Crosslinking

Recombinant human TGF-β1 was obtained from Arlen Thomason (Amgen Corp.) and Hideya Ohashi (Kirin Brewery Co.). TGF-β1 was iodinated using the chloramine T method according to Frolik et al. (1984). Cross-linking experiments were performed as previously described (Ichijo et al., 1990). Briefly, cells in 6-well plates were washed with binding buffer (phosphate-buffered saline containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$ and 1 mg/ml bovine serum albumin (BSA)), and incubated on ice in the same buffer with ^{125}I-TGF-β1 in the presence or absence of excess unlabeled TGF-β1 for 3 h. Cells were washed and cross-linking was done in the binding buffer without BSA together with 0.14 mM of disuccinimidyl suberate (DSS; Pierce Chemical Co.) for 15 min on ice. The cells were harvested by the addition of 1 ml of detachment buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 0.3 mM PMSF). The cells were pelleted by centrifugation, then resuspended in 50 μl of solubilization buffer (125 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.3 mM PMSF, 1% Trasylol) and incubated for 40 min on ice. Another centrifugation was done and supernatants were subjected to analysis by SDS-gel electrophoresis using 4-15% polyacrylamide gels, followed by

autoradiography. For immunoprecipitation of the cross-linked complexes, cells in 25 cm^2 flasks were used. The supernatants obtained by cross-linking were incubated with 7 µl of preimmune serum or VPN antiserum in the presence or absence of 10 µg of peptide for 1.5 h at 4°C. Immune compexes were then added with 50 µl of protein A-Sepharose slurry and incubated for 45 min at 4°C. The protein A-Sepharose beads were washed four times with the washing buffer, one time with distilled water, and the samples were analyzed by SDS-gel electrophoresis using 4-15% polyacrylamide gradient gels and autoradiography.

Northern Blot Hybridization

A Northern blot filter with mRNAs from different human tissues was obtained from Clontech. The filter was hybridized with a ^{32}P-labeled probe at 42°C overnight in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 0.1% SDS, 50 mM sodium phosphate, pH 6.9, and 0.1 mg/ml salmon sperm DNA. An EcoR1 fragment of 980 bp of the full-length cDNA clone, corresponding to the C-terminal part of the kinase domain and 3' untranslated region (nucleotides 1259-2232 in Fig. 1B) was radiolabeled by the Megaprime DNA labeling system, and used as a probe. The filter was washed two times in 0.5 x SSC, 0.1% SDS at 55°C for 15 min.

PAI-1 Assay

PAI-1 assay on wild type Mv1Lu cells and mutant transfected or not with the ALK-5 cDNA was performed as described previously (Laiho et al., 1991b). Briefly, cells were added with or without 10 ng/ml of TGF-β1 for 2 h in serum-free MCDB 104 without methionine. Thereafter, cultures were labeled with [^{35}S]methionine (40 µCi/ml) for 2 h. The cells were removed by washing on ice once in PBS, three times in 10 mM Tris-HCl (pH 8.0), 0.5 % sodium deoxycholate, 1 mM PMSF, two times in 2 mM Tris-HCl (pH 8.0), and once in

PBS. Extracellular matrix proteins were extracted by scraping into the SDS-sample buffer containing DTT, and analyzed by SDS-gel electrophoresis followed by fluorography using Amplify. Gels were analyzed by PhosphorImager (Molecular Dynamics). PAI-1 was identified as a characteristic 45 kd band (Laiho et al., 1991b).

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Figure Legends

Figure 1. cDNA Cloning and Sequence of ALK-5

(A) Structure of the ALK-5 cDNA clone EMBLA. Boxes represent the coding region; filled box indicates the signal peptide, shaded box the transmembrane domain, and hatched box the intracellular kinase domain. The EcoRI cleavage sites are also indicated. (B) The nucleotide and deduced amino acid sequences of ALK-5. Nucleotides and deduced amino acids are numbered to the right, starting with the proposed initiating Met codon. The N-terminal hydrophobic signal sequence and transmembrane domain are overlined (thin lines), and the potential N-glycosylation site is underlined (thick line). Cysteine residues in the extracellular domain are boxed and the stop codon which ends the open reading frame is marked with an asterisk. The beginning and the end of the kinase domain are indicated by arrows. Half arrows indicate the regions in the kinase domain that were used for construction of the primers for PCR. The in-frame stop codon found in the 5' untranslated region is underlined with a thin line. (C) Comparison of the amino acid sequences of the ALK-5 (TβR-I) and the human TGF-β type II receptor (TβR-II) (Lin et al., 1992). Alignment was perfomed with the Clustal computer alignment program (Higgins and Sharp, 1989), with some manual adjustment. Identical amino acids are boxed, and cysteine residues in the extracellular domain are shaded. The hydrophobic leader sequence and transmembrane domain of the ALK-5/TβR-I are overlined. The borders of the kinase domain are indicated by arrows and the kinase inserts are underlined by bold lines. The kinase subdomains are shown with roman numerals according to Hanks et al. (1988).

Figure 2. Transient Expression of ALK-5 in COS Cells

Control COS-1 cells and COS-1 cells transfected with the pSV7d expression vector containing the ALK-5 cDNA were metabolically labeled with

[^{35}S]methionine and [^{35}S]cysteine for 6 h. The cell lysates were then subjected to precipitation using preimmune serum (pre) or the VPN antiserum (immune). Blocking of the immune serum was performed with 10 μg of peptide (block). Enzymatic deglycosylation of ALK-5 was performed by incubating the immunoprecipitates with 0.5 U of endoglycosidase F (endo F) at 37°C for 24 h. Precipitates were analyzed by SDS-gel electrophoresis and fluorography. Specific bands are indicated by arrows.

Figure 3. Binding of ^{125}I-TGF-β1 to PAE/TβR-I Cells

(A) The parental PAE cells and PAE/TβR-I cells were affinity labeled with ^{125}I-TGF-β1, in the presence or absence of excess unlabeled TGF-β1 (cold TGF-β). After cross-linking with DSS, samples were analyzed by SDS-gel electrophoresis and autoradiography before (antiserum -) or after immunoprecipitation by the VPN antiserum (im) or preimmune serum (pre). (B) The PAE/TβR-I cells were affinity cross-linked using ^{125}I-TGF-β1, and samples were analyzed by SDS-gel electrophoresis and autoradiography before (antiserum -) or after immunoprecipitation by the antiserum against ALK-5 (VPN) or against the TGF-β type II receptor (DRL).

Figure 4. Characterization of the ALK-5 Protein in PAE/TβR-I Cells

(A) Enzymatic deglycosylation of the cross-linked complexes by endoglycosidase F. The PAE/TβR-I cells were labeled with ^{125}I-TGF-β1 and cross-linked with DSS. The samples were then treated with 0.5 U of endoglycosidase F (endo F) before or after immunoprecipitation (IP) by the VPN antiserum, and analyzed by SDS-gel electrophoresis and autoradiography. (B) Binding of ^{125}I-TGF-β1 to DTT treated PAE/TβR-I cells. The PAE/TβR-I cells were treated with 1 mM DTT at 37°C for 8 min in the binding buffer without BSA, then incubated with ^{125}I-TGF-β1 followed by cross-linking with DSS. The cross-linked complexes were analyzed by SDS-

gel electrophoresis and autoradiography before or after immunoprecipitation (IP) by the VPN antiserum.

Figure 5. Northern Blot Analysis of ALK-5 mRNA

A blot with mRNA prepared from different human tissues (Clontech) was hybridized with a radiolabeled 980 bp internal EcoRI fragment of the ALK-5 cDNA. Each lane contained 2 μg of polyadenylated RNA from the indicated tissues. An autoradiography of the hybridized blot is shown.

Figure 6. Expression of the type I and type II TGF-β receptor complexes in different cell types.

(A) Wild type Mv1Lu and the R mutant clone (clone 4-2) were affinity labeled with ^{125}I-TGF-β1 in the presence or absence of excess unlabeled TGF-β1 (cold TGF-β1). After cross-linking with DSS, samples were analyzed by SDS-gel electrophoresis and autoradiography before (antiserum -) or after immunoprecipitation using the VPN antiserum or the DRL antiserum. (B) Human foreskin fibroblasts (AG1518), human lung adenocarcinoma cells (A549; American Type Culture Collection), and human oral squamous cell carcinoma cells (HSC-2; Ichijo et al., 1990) were subjected to affinity cross-linking and samples were analyzed by SDS-gel electrophoresis before (antiserum -) or after immunoprecipitation using the VPN antiserum or the DRL antiserum. The gels were exposed to Hyperfilm-MP (Amersham) for autoradiography (AG1518 and A549 cells) or analyzed by PhosphorImager (HSC-2 cells).

Figure 7. Induction of PAI-1 in the R mutant after transfection of the ALK-5 cDNA.

Wild type Mv1Lu cells or the R mutant clone transfected or not with the ALK-5 cDNA were incubated with or without 10 ng/ml of TGF-β1. After 2 h of

incubation, the cells were metabolically labeled with [^{35}S]methioneine for additional 2 h. Extracellular matrix proteins were recovered, and the production of the 45 kd PAI-1 protein was analyzed by SDS-gel electrophoresis followed by fluorography.

CLAIMS

1. An isolated nucleic acid molecule which codes for, or is complementary to a nucleic acid molecule which codes for, a TGF-ß-type I receptor.

2. A nucleic acid molecule comprising a heterologous sequence which codes for, or is complementary to a nucleic acid molecule which codes for, a TGF-ß-type I receptor.

3. A molecule according to claim 1 or claim 2, wherein the receptor is as shown in Fig. 1(C), or a functionally-equivalent part thereof.

4. A DNA molecule according to any preceding claim.

5. A molecule according to any preceding claim, which additionally comprises, operably associated with the coding sequence, a sequence adapted to allow expression of the receptor.

6. A host comprising a molecule according to claim 5, which is capable of expressing the receptor.

7. An isolated protein having TGF-ß-type I receptor functionality.

8. An isolated protein having an amino-acid sequence corresponding to part or all of the amino-acid sequence of a TGF-ß-type I receptor, wherein said protein has at least one of the following characteristics:

(i) serine/threonine kinase activity;

(ii) TGF-ß binding activity; and

(iii) TGF-ß-type II receptor interaction.

9. A protein according to claim 7 or claim 8, having all or part of the sequence shown in Fig. 1(C).

10. An antibody to a protein as defined in any of claims 7 to 9.

11. A product according to any preceding claim, for therapeutic or diagnostic use.

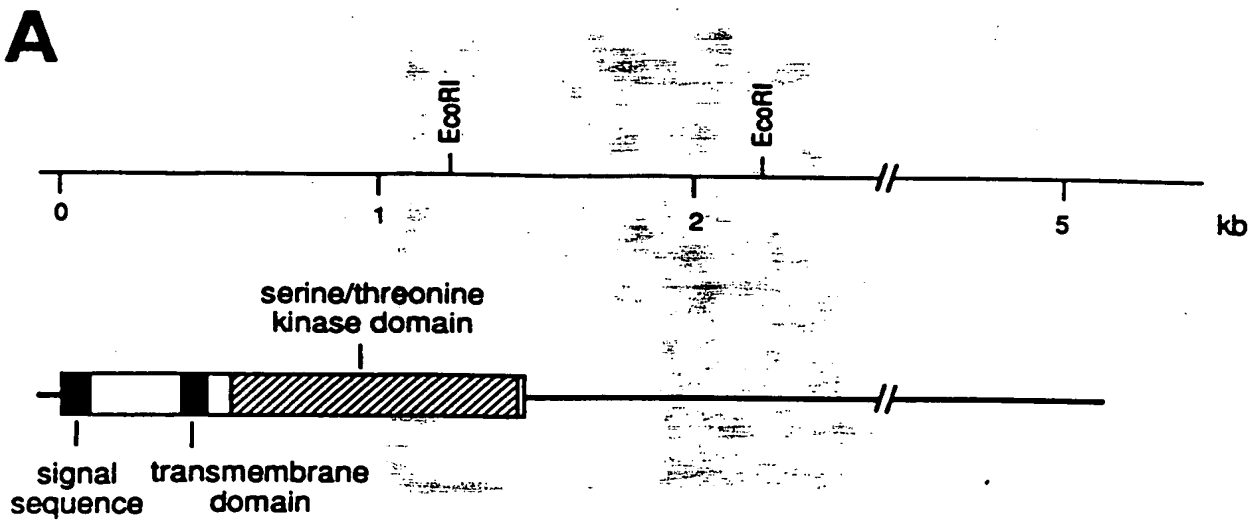

Fig 1 A
Franzén et al

B

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Fig 1B
Franzén et al

C

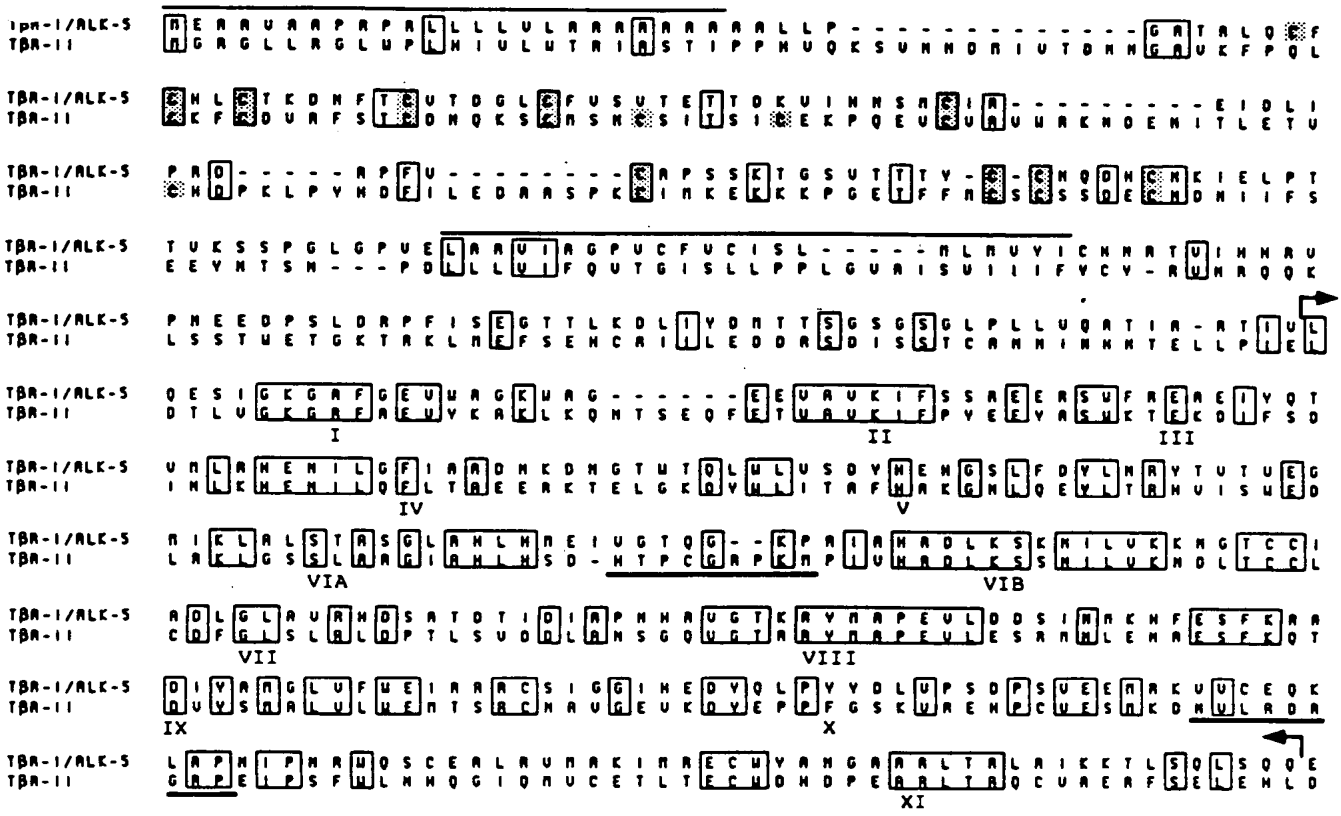

Fig 1C

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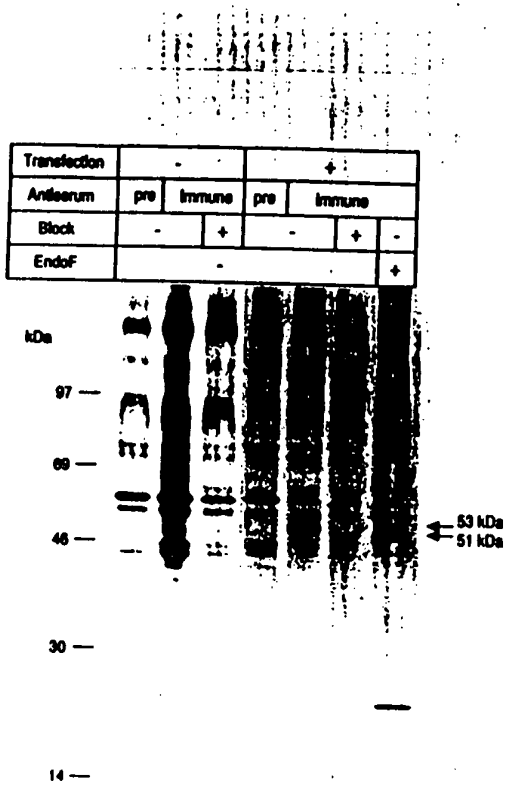


Fig. 2

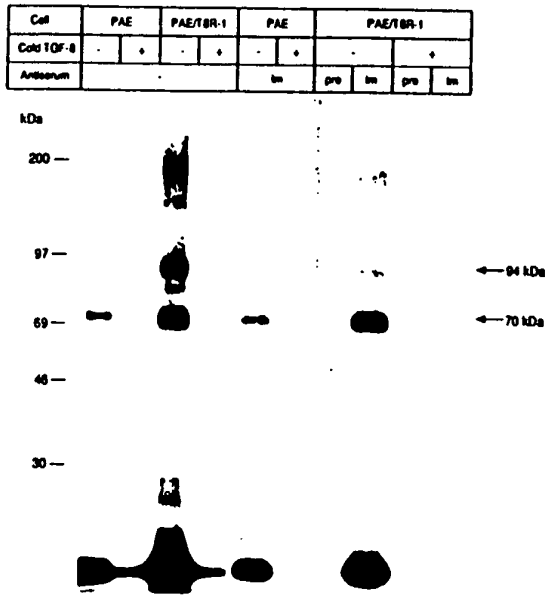


Fig. 3A

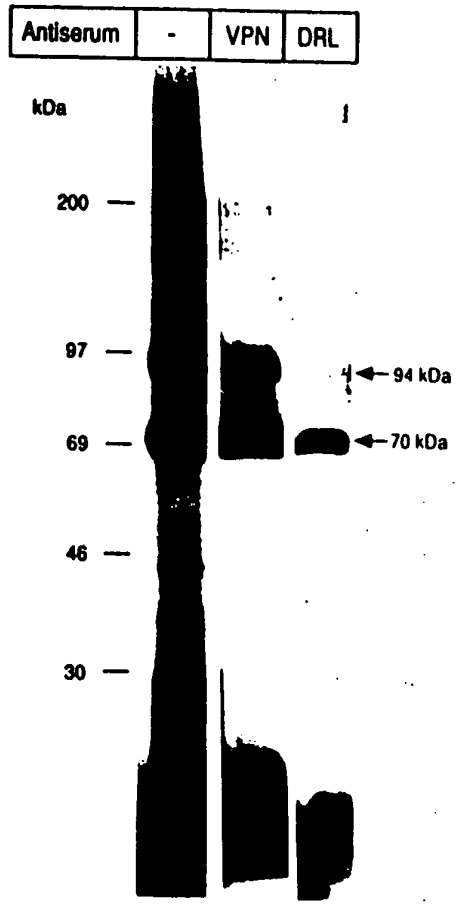


Fig. 3B

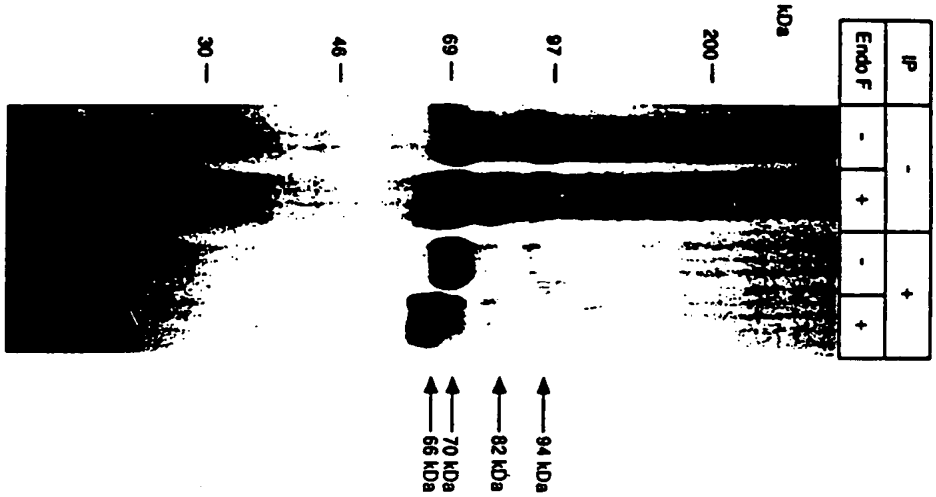


Fig 4A

Fig. 4B

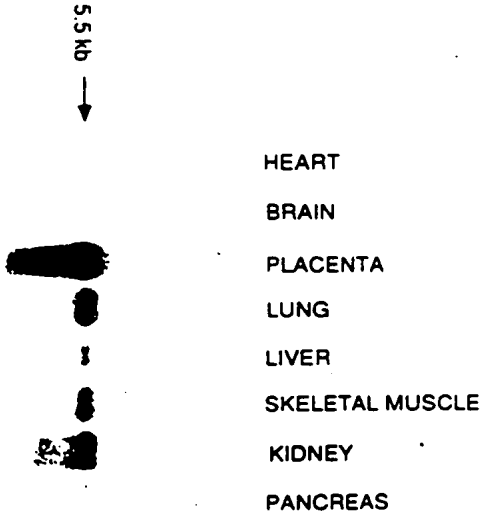
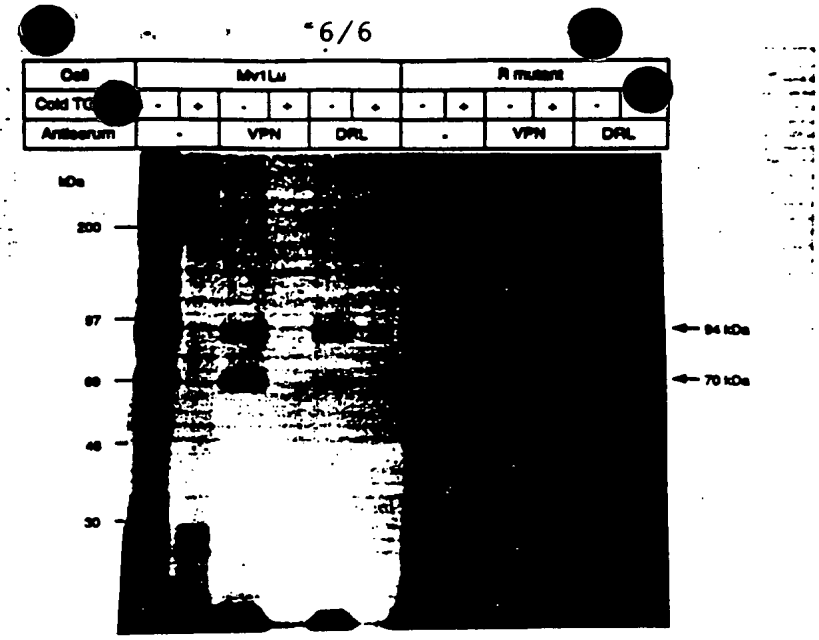


Fig. 5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Antiserum
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kDa
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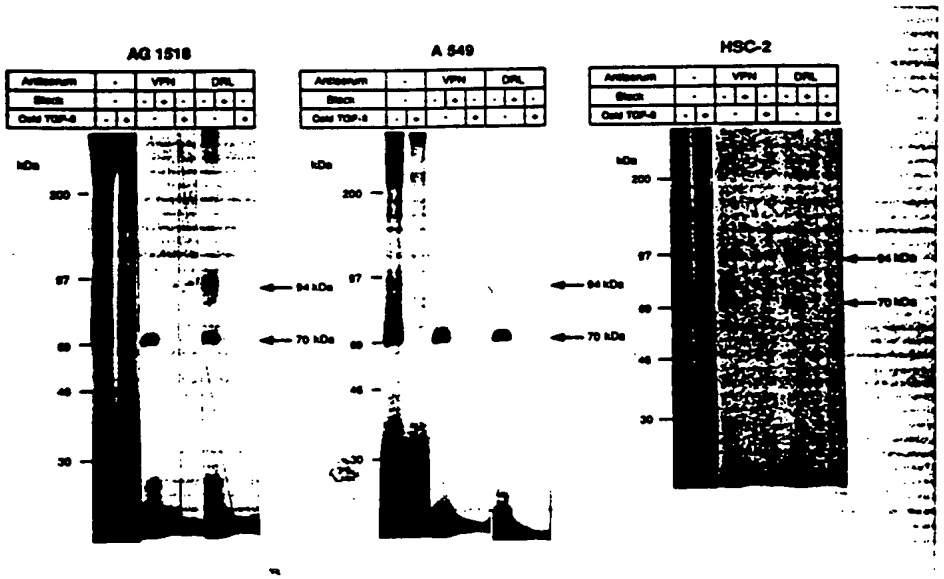
46
30
94 kDa
70 kDa

Fig. 6A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Block
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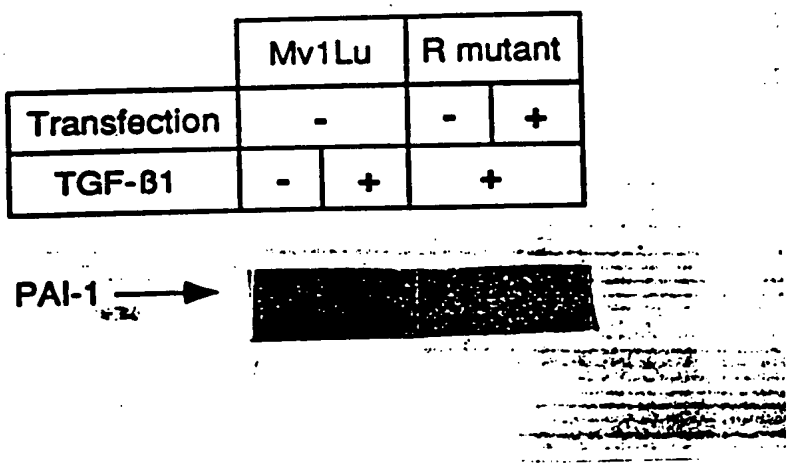
Fig. 6B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R mutant
Transfection
TGF-β1
PAI-1